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Original Article

Molecular and morphophysiological responses cocoa leaves with different concentrations of anthocyanin to variations in light levels

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ABSTRACT

Theobroma cacao gives higher yield when cultivated in full sun under irrigation system with fertilization, because is a species little conservative in relation the efficiency of water use. On the other hand, *T. cacao* is usually cultivated under shade conditions in '*Cabruca*' and agroforestry systems but with low yield. It has been observed under field conditions that the genotypes of *T. cacao* with high concentration of anthocyanins in young leaves are more tolerant to high irradiance stress when grown in full sun. The accumulation of flavonoids or other UV-absorbing compounds in the leaf epidermis is one of the most important mechanisms to screening out UV-B radiation. The aim of this study was to evaluate the effects of different levels of light on three clonal cacao genotypes ('Catongo', SCA-6 and SJ-2), contrasting in relation to accumulation of anthocyanin levels in young leaves, by evaluations of photosynthesis, activity of guaiacol peroxidase (GPX), chloroplastid pigment contents and vacuolar (anthocyanins) flavonoids contents, anatomical characteristics and gene expression of the leaf. In summary, (i) the anthocyanins contents in leaf level did not provide protection against photoinhibition in *T. cacao*, (ii) the 'Catongo' and SJ-2 genotypes showed greater phenotypic plasticity to the morphology and the chloroplastidic pigment contents in the leaf, while the SCA-6 genotype allocated more in the flavonoids content and hsp70 gene expression; (iii) the relative expression of the genes *psbA* and *psbO*, did not vary between genotypes under irradiance stress.

1. Introduction

Cacao, *Theobroma cacao* L., is one of the world's important agricultural export commodities, with it being cultivated mainly for chocolate production (Almeida and Valle 2007). Traditionally, cacao is cultivated under the shade of a selectively thinned forest. In the Atlantic coastal forests of the states of Bahia and Espirito Santo, Brazil, around 4% of the world and 75% of the Brazilian cacao output is obtained using a system locally called '*Cabruca*' (Lobão et al., 2007). This system is a special kind of agroforestry in which the understorey is drastically suppressed to introduce cacao, and the density of upper storey trees is reduced. Cacao is also intercropped around the world in planned systems with other species of economic value crops such as *Areca catechu*, *Cocos nucifera* (Alvim and Nair 1986; Daswir and Dja'far, 1988; Abbas and Dja'far, 1989), *Hevea brasiliensis*, *Syzigium aromaticum*, *Cinnamomum zeylanicum*, *Erythrina fusca* (Alvim, 1989a,b), *Bactris gasipaes* (Almeida et al., 2002) and other Amazonian species (Brito et al., 2002). However, *T. cacao* cultivation has also been observed in full sun, in irrigation systems with fertilization. Under full system cacao requires vast amounts of water to achieve high yields (fruit production) in comparison to "Cabruca" and agroforestry cultivation systems (Almeida and Valle, 2007).

Sunlight provides the energy required for plant photosynthetic reactions. In contrast, damage can be caused with excessive exposure to high sun light, while inadequate sun light can limit plant growth and development (Lambers et al., 1998). However, plants have developed sophisticated stress response mechanisms resulting from high light intensities. Low light levels can cause stresses on plants, due to decreased carbon dioxide (CO_2) assimilation rates, decreased carbohydrate production and decreased growth and development. On the other side, high light levels can damage the photosynthetic machinery (Lambers et al., 1998), known as photoinhibition which may be divided into two types: (i) dynamic photoinhibition, which shows a reduction in quantum efficiency of the photosystem 2 (PS2) reversible, accompanied

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by a significant increase in the thermal dissipation of excess energy absorbed, indicating that the decrease in the photochemical efficiency should if, in part, to mechanisms photoprotections; and (ii) chronic photoinhibition which occurs when excess absorbed light generates a series of highly reactive oxygen intermediates (ROIs) which can cause serious problems for the photosynthetic machinery (Mittler, 2002) with damage to lipid membrane constituents, pigments and cofactors critical protein subunits, especially the D1 protein, resulting in complete inactivation of the photo-oxidative reaction center (Apel and Hirt, 2004). This damage, if not repaired, lead to a decrease of the photochemical efficiency, which can be characterized by an irreversible decrease the Fv/Fm ratio (quantum efficiency of the PS2) (Hideg and Murata, 1997).

Plants have developed various strategies to cope with adverse conditions, such as plasticity in terms of acclimation to full sunlight and shade, as well as photoprotective mechanisms designed to dissipate excess energy (Demmig-Adams and Adams, 1992). Differences in leaf morphology, anatomy and physiology have been documented for species that are adapted to shaded or full sun environments (Boardman 1977; Björkman 1981; Givnish 1988). Structural foliar changes can also be adaptable, for example, the inactivation of the reaction center of the PS2 (Öquist et al., 1992). Changes in existing leaf physiology and the production of new leaves, that are morphologically and physiologically suitable to the light environment, are acclimation response components (Wyka et al., 2007).

Additionally, plants have developed enzymatic and non-enzymatic protection mechanisms in response to oxidative stress, which are triggered by variations in light intensity. The non-enzymatic mechanism includes metabolites such as anthocyanins and other types of flavonoids (Marchese et al., 2008; Pietrini et al., 2002); while the enzyme mechanism is composed by superoxide dismutase, by catalases and peroxidases, which subsequently act to detoxify H_2O_2 (Apel and Hirt 2004). The accumulation of flavonoids or other UV-absorbing compounds in the leaf epidermis is one of the most important mechanisms to screening out UV-B radiation. The accumulation of anthocyanins in leaf level reduces the oxidative stress, induced by exposure to ultraviolet-B (UV-B), and the photoinhibitory damages (Fini et al., 2011). It has been observed under field conditions that the genotypes of *T. cacao* with high content of anthocyanins, in young leaves, are more tolerant to stress promoted by the high light intensity when grown in full sun.

The species *T. cacao*, due to it being a preferentially allogamous species, has a very large variety of genotypes, with striking phenotypic differences. The main objective of this study was to evaluate the effects of different light levels on three clonal genotypes of cocoa (‘*Catongo*’, SCA-6 and SJ-2), which were different in terms of the presence of anthocyanins at the foliar level, through photosynthesis evaluations, of guaiacol peroxidase activity (GPX), of the content of chloroplastidic and vacuolar pigments (anthocyanins), of flavonoid content, by anatomical analysis and of gene expression at the foliar level.

2. Materials and methods

2.1. Growth conditions and plant material

The experiment was performed from September/2010 to November/2011 at the campus of State University of Santa Cruz (UESC), located in the city of Ilhéus, Bahia, Brazil (14°47′ S, 39°10′ W). Three clonal genotypes of cocoa were used, which varied in terms of their anthocyanin content in young leaves (‘*Catongo*’ − no anthocyanins, SCA-6–low content of anthocyanins and SJ-2–high content of anthocyanins). The genotype ‘Catongo' is a natural mutant anthocyanins, with monogenic or qualitative inheritance for this character, self-compatible and highly productive; with the young leaves (Fig. 1), and seeds devoid of this vacuolar pigment, whose seeds are white; besides being intolerant to witches' broom disease (Bartley, 2005). However, SCA-6 is a wild type genotype, self-incompatible, has small fruits and seeds,; young leaves (Fig. 1), fruits and seeds are slightly red by the presence of vacuolar anthocyanins, as well as having alleles for tolerance to witches' broom (Bartley, 2005). SJ-02 genotype is an auto-compatible and highly productivity clonal cultivar that has young leaves (Fig. 1), fruits and seeds intense red coloration due to the high concentration of anthocyanins in vacuolar constituent cells of the tissues and organs. The clonal genotypes were obtained by rooting 16-cm-long stem cuttings from plagiotropic branches at the beginning of secondary growth, containing the apical bud, three auxiliary buds and three leaves. The bottoms of the cuttings (± 3 cm) were dipped into chemically inert talcum powder containing indol-3-butyric acid (IBA) at 4 g kg^{-1}. Afterwards, each cutting was transferred to a 288-cm^3 tube-like, black plastic pot containing organic substrate (turf + grinded Pinus sp. barks and grinded coconut fiber at 1:1 ratio) enriched with macro and micronutrients, according to the recommendations for cacao. The planted pots were left at a nursery with 50% sunlight cover and irrigated by microaspersion. After 4 months of growth these rooted cuttings were transplanted to 12 L plastic pots, containing soil as substrate, and grown in 100%, 50% and 5% of full sunlight, during seven months. The different conditions of light levels simulate the main forms of cocoa cultivation in the world (100% − monoculture in full sun; 50% − agroforestry systems with species of economic value such as *Areca catechu*, *Cocos nucifera*, *Hevea brasiliensis*, *Syzigium aromaticum*, *Cinnamomum zeylanicum*, with different spacing between plants; and 5% − an agroforestry system, known as ‘Cabruca’, is a main cropping system invariably adapted for cultivation of cacao in Brazil, in this system of management, cacao is grown under the shade of native species of the Atlantic Forest. During the experimental period, the photosynthetic photon flux density (PPFD) was monitored in environments with 100% (full sun), 50% and 5% light levels, at the clonal saplings' extremities, with the use of a *S-LIA-M003* luminous radiation sensor coupled to a micro climatological station: *Hobo Micro Station Data Logger* (*Onset*, *USA*) (Supplementary material 1). Precipitation was monitored using a pluviometer. The mean values (± EP) for air temperature, relative humidity and pluviometric precipitation, obtained during the experimental period, were 24.1 ± 0.4 °C, 87.8 ± 1.6% and 457.8 ± 12.5 mm, respectively.

2.2. Leaf gas exchange

During the experimental period, the net photosynthetic rate per unit of leaf area (P_N), stomatal conductance to water vapor (g_s) and leaf transpiration (E) were measured, between 08:00 and 9:00 a.m., on a mature and completely expanded leaf from the end the orthotropic apex axis. Five plants per treatment were assessed using a LI-6400 portable photosynthesis system (Li-Cor, Nebraska, USA) equipped with a 6400–02 B RedBlue artificial light source. For the leaf gas exchange measurements, the artificial light source of the system was adjusted to provide a photosynthetic photon flux density (PPFD) of 700 μmol m^{-2} s^{-1}. To save each reading, the minimum pre-established time for reading stabilization was 60 s and the maximum 120 s. Also, the reading was saved if the coefficient of variation (CV) for the measurements was less than 0.3%. In addition to PPFD, temperature and atmospheric CO_2 within the leaf chamber were maintained constant at 26 °C and 380 μmol (CO_2) mol^{-1}, respectively.

2.3. Fluorescence emission

The Chl fluorescence emission was measured simultaneously on the same leaves (n = *5*) used for the gas-exchange measurements, with a leaf chamber fluorometer LI 6400-40, a LED-based fluorescence accessory for the portable photosynthesis system LI-6400 *(*LI-COR Bioscience Inc., *Lincoln, NE, USA)*.

To assess the Chl fluorescence emission in dark-adapted leaves, the leaf tissue was placed in standard *LI-COR* leaf clips for 30 min on each leaf prior to each making each measurement. Following dark-adaptation, the leaf tissue was illuminated with a weak-modulated measuring